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Cancer Patient T Cells Genetically Targeted to Prostate-Specific Membrane Antigen Specifically Lyse Prostate Cancer Cells and Release Cytokines in Response to Prostate-Specific Membrane Antigen

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Abstract

The expression of immunoglobulin-based artificial receptors in normal T lymphocytes provides a means to target lymphocytes to cell surface antigens independently of major histocompatibility complex restriction. Such artificial receptors have been previously shown to confer antigen-specific tumoricidal properties in murine T cells. We constructed a novel ζ chain fusion receptor specific for prostate-specific membrane antigen (PSMA) termed Pz-1. PSMA is a cell-surface glycoprotein expressed on prostate cancer cells and the neovascular endothelium of multiple carcinomas. We show that primary T cells harvested from five of five patients with different stages of prostate cancer and transduced with the Pz-1 receptor readily lyse prostate cancer cells. Having established a culture system using fibroblasts that express PSMA, we next show that T cells expressing the Pz-1 receptor release cytokines in response to cell-bound PSMA. Furthermore, we show that the cytokine release is greatly augmented by B7.1-mediated costimulation. Thus, our findings support the feasibility of adoptive cell therapy by using genetically engineered T cells in prostate cancer patients and suggest that both CD4⁺ and CD8⁺ T lymphocyte functions can be synergistically targeted against tumor cells.

Keywords: gene therapy, prostate cancer, immunotherapy, retroviral-mediated gene transfer, costimulation.

Introduction

The infusion of T lymphocytes as a therapeutic modality against cancer is based on the premise that tumor-reactive lymphocytes can be expanded *ex vivo* and retain their antitumoral functions upon reinfusion *in vivo*. While autologous adoptive cell therapies have shown some beneficial effects in cancer patients with solid tumors [1], they are limited by several biological obstacles that include difficulties in generating tumor-specific lymphocytes and the likely occurrence of anergy and/or cell death facing the infused lymphocytes [2,3]. In principle, these shortcomings could be in part remedied by genetically programming the T cells to

recognize tumor cells [4] and to survive in a proapoptotic environment [5]. Artificial T-cell receptors (TCR) that incorporate the antigen-specific moiety of an immunoglobulin, expressed as a single chain antibody (scFv), provide a means to target antigens independently of major histocompatibility complex (MHC) expression. Such receptors are therefore expected to target T cells to tumor cells irrespective of the patient's MHC haplotype and of MHC expression by tumor cells. The targeted antigen must be a cell surface antigen, ideally absent from normal tissues or at least less accessible therein. To elicit T-cell functions, recognition of antigen must be followed by an activation signal transduced via the receptor's cytoplasmic domain. Artificial TCRs that include the activation domain of the CD3-associated ζ chain have been shown to confer tumoricidal properties to murine T cells [4]. However, their activity in cancer patient lymphocytes has not yet been reported.

We have created an scFv-based ζ chain fusion receptor, termed Pz-1, that is specific for prostate-specific membrane antigen (PSMA). PSMA is a cell-surface glycoprotein expressed on prostate epithelial cells whose expression is increased in advanced prostate cancer [6–8]. PSMA is thus an attractive target molecule for immunotherapy in patients with advanced prostate cancer, for which there is no effective treatment. Moreover, PSMA is found in the neovasculature of numerous solid tumors including renal cell, urothelial, colon and breast carcinomas as well as melanoma and sarcoma [8]; Chang et al., manuscript submitted, and thus lymphocyte targeting to PSMA is potentially of broader use. Taking advantage of highly efficient gene transfer in human primary T cells [9], we therefore examined the function of ζ chain fusion receptors in T cells from prostate cancer pa-

Abbreviations: CTL, cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; IFN, interferon; IL-2, interleukin-2; MHC, major histocompatibility complex; NTP, truncated form of the human low affinity nerve growth factor receptor; PBL, peripheral blood lymphocyte; PE, phycoerythrin; PSMA, prostate-specific membrane antigen; Pz-1, PSMA-specific J591- ζ chain fusion receptor; scFv, single chain antibody (fragment variable); TCR, T-cell receptor.

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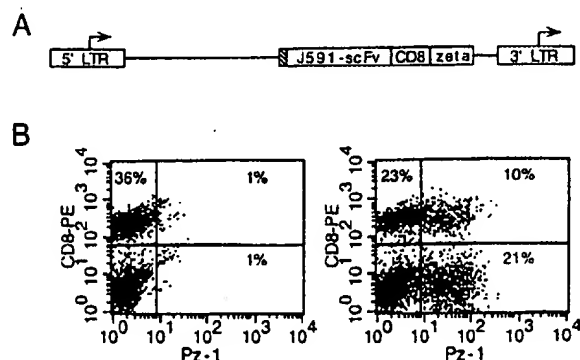


Figure 1. Pz-1 vector structure and expression in primary human T cells. (A) Schematic diagram of the Pz-1 retroviral vector (Gong et al., manuscript in preparation). J591-scFv: PSMA-specific scFv derived from the monoclonal antibody (mAb) J591 [8]; CD8: CD8 hinge and transmembrane domains; zeta: T-cell receptor ζ chain cytoplasmic domain; LTR: long terminal repeat; hatched box: CD8 leader sequence; arrows indicate start of transcription. (B) FACS (Becton Dickinson & Co) analysis of transduced primary T cells. PBL harvested from cancer patients were transduced as previously described [9] and stained 3 days after retroviral infection to enumerate the fraction of Pz-1⁺ CD4⁺ and Pz-1⁺ CD8⁺ lymphocytes. Lymphocytes were incubated with an FITC-conjugated idiotype-specific antiserum, which recognizes the J591-derived scFv. After washing and incubation with 10% normal mouse serum, lymphocytes were stained with a PE-conjugated anti-CD8 mAb. Y axis: CD8^{PE}, X axis: Pz-1 signal. Left panel: Mock transduction; right panel: Pz-1 transduction. The percentage of cells present in each quadrant is indicated.

tients. We demonstrate that Pz-1 is capable of directing peripheral blood lymphocytes (PBL) to specifically recognize and lyse prostate cancer cells expressing PSMA. We show that cytotoxicity against PSMA-positive target cells was obtained in the PBL of 5 out of 5 patients. Furthermore, we assessed the cytokine response of T cells transduced with Pz-1 upon contact with PSMA-positive cells and examined whether CD28-mediated costimulation synergized with Pz-1 signaling to augment the cytokine release of PSMA-specific human T cells. These observations have important implications for the generation and use of genetically engineered autologous lymphocytes to treat cancer.

Materials and Methods

Cell Lines

Retroviral producer cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum (FCS) as described [9]. Prostate cancer cell lines, PC-3 and LNCaP, and murine thymoma EL4 cells were maintained in RPMI

with 10% FCS. NIH3T3 fibroblasts (American Type Culture Collection, Rockville, MD) were maintained in DMEM with 10% donor calf serum (DCS, Hyclone, Logan, UT). All media contained penicillin (100 U/mL) and streptomycin (100 μ g/mL).

PBL Collection and Transduction

PBL harvest and retroviral infection were carried out as described elsewhere [9]. After retroviral infection, PBL were maintained in RPMI with 10% human serum (Pel-Frez, Brown Deer, WI) and 20–50 IU/mL interleukin-2 (IL-2, Chiron, Emeryville, CA).

Flow Cytometry

Pz-1 artificial T-cell receptor expression in PBL was evaluated by incubating the transduced lymphocytes with fluorescein isothiocyanate (FITC)-conjugated idiotype-specific antiserum, which recognizes the prostate-specific membrane antigen (PSMA)-scFv. PSMA expression in target or feeder cells was determined with the J591 mAb [8]. B7.1 expression in the feeder cells was determined with mAb BB1 (PharMingen, San Diego, CA), specific for human B7.1.

PBL Cocultivation

For cocultivation with fibroblasts, 10^5 NIH3T3 cells per well were irradiated and cultured in AIM-V serum free media (Gibco-BRL, Gaithersburg, MD) supplemented with 5% DCS. The following day, 10^6 T cells per well were added. Supernatants were analyzed at 24, 48, and 72 hours after start of the cocultivation for IL-2 and interferon (IFN)- γ with ELISA kits (Genzyme, Cambridge, MA).

Cytotoxicity Assays

Cytotoxic T lymphocyte (CTL) assays were performed 4 to 14 days after PBL transduction. Transduced PBL were incubated for 4 hours at different effector to target (E:T) ratios with 10^4 chromium⁵¹-labeled target cells at 37°. Triplicate supernatants were counted using an automated gamma counter. Percent specific ⁵¹Cr release was calculated by (⁵¹Cr release – spontaneous release)/(maximum ⁵¹Cr release – spontaneous release) \times 100.

Results

Pz-1 Transduction and Expression in Human Primary T Lymphocytes

The PSMA specific ζ chain chimeric receptor Pz-1 was cloned into a retroviral vector (Figure 1A) and transduced in

Table 1. Prostate Cancer Patient Demographics.

	Current Age	Time Since Diagnosis (mo)*	GG	Stage at Diagnosis	Treatment	Current Stage	Current PSA (ng/mL) [†]
Patient 1	67	19	3 + 3	T1c	Observation	T1c	10
Patient 2	85	251	5 + 4	T3	Hormonal	T4M +	501
Patient 3	54	8	4 + 5	T1c	RRP	pT3CN0Mx rising PSA	4

*Time elapsed between PBL harvest and date of diagnosis of prostate cancer.

[†]Reference range of PSA, 0–4 ng/mL. Rising PSA is biochemical evidence of residual prostate cancer (site of residual disease unknown).

(GG: Gleason grade of most recent prostate cancer pathology; RRP: radical retropubic prostatectomy).

Table 2. Gene Transduction Efficiency in T Cells from Prostate Cancer Patients Measured on Day of CTL Assay.

Patient	%TR	%CD8	%CD56	%CD16
Pt 1 Pz-1	23	15	< 1	< 1
Pt 2 Pz-1	40	13	< 1	< 1
Pt 3 Pz-1	49	44	5	< 1
Pt 1 NTP	18	15	ND	ND
Pt 2 NTP	37	13	ND	ND
Pt 3 NTP	27	44	ND	ND

(%TR, percentage of gene transduction measured as shown in Figure 2B; %CD8, percentage of CD8⁺ T cells; %CD56, percentage of CD56⁺ cells; %CD16, percentage of CD16⁺ cells; ND, not done).

patient PBL with gibbon ape leukemia virus envelope-pseudotyped virions as previously described [9]. PBL were harvested from five prostate cancer patients in a variety of clinical stages, including the three representative cases shown in Table 1. Gene transfer efficiency, monitored by fluorescence-activated cell sorter (FACS; Becton Dickinson & Co) and analysis with a Pz-1 idiotype-specific antiserum (Figure 1B), varied between 20% and 50% in both CD8⁺ and CD4⁺ cells (Table 2). CD8⁺ cells represented 13% to 44% of the different cultures, whereas cells expressing the CD56 and CD16 markers found on natural killer (NK) cells represented fewer than 1% of the cells in two cases and 5% in the third. Gene transfer efficiency of the marker NTP [9], a mutated human low-affinity nerve growth factor receptor used as control (Table 2).

Pz-1 Directs PSMA-Specific Cytotoxicity in T Cells From Prostate Cancer Patients

CTL assays were first performed on LNCaP, a human prostate cancer cell line that abundantly expresses PSMA [6,8] (Figure 2A). As shown in Figure 2B, the PBL of all three patients were able to recognize and lyse ⁵¹Cr-labeled LNCaP after transduction with Pz-1 but not NTP. PC-3, a human prostate cancer cell line normally negative for PSMA, and EL-4, a murine thymoma, were transduced with a retrovirus encoding PSMA (Latouche et al., unpublished observations). PSMA expression was confirmed by Western

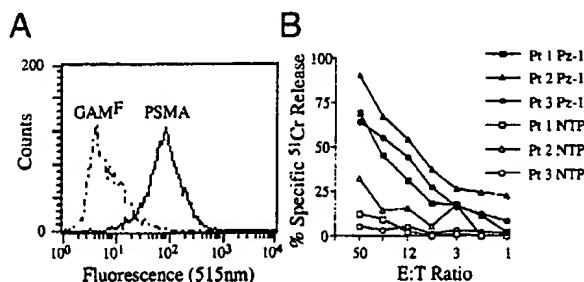


Figure 2. Pz-1-transduced prostate cancer patient T lymphocytes lyse LNCaP cells. (A) LNCaP cell expression of PSMA. FACS (Becton Dickinson & Co) analysis of wild type LNCaP cells. Cells were stained with mAb J591 followed by FITC-conjugated goat anti-mouse (GAM-FITC) antibody. Y axis: cell count, X axis: fluorescence (515 nm). PSMA: PSMA signal, GAMF: GAM-FITC antibody alone signal. (B) Pz-1 directed cytotoxicity against LNCaP cells. PBL from three patients (Tables 1 and 2) were transduced with either Pz-1 or NTP [9]. Transduced PBL were used in CTL assays with LNCaP target cells. Percentage of specific lysis is represented on the Y axis, effector to target ratio on the X axis.

blot analysis (data not shown) and by FACS (Becton Dickinson & Co) analysis with different monoclonal antibodies (mAbs) specific for PSMA. Figure 3A shows the FACS (Becton Dickinson & Co) analysis of PSMA expression in transduced PC-3 and EL-4 cells. Staining of the parental PC-3 or EL-4 shows no detectable PSMA expression. Transduced PC-3 cells (PSMA⁺) were of comparable brightness to LNCaP cells (Figure 2A).

T-cell populations transduced with Pz-1 but not NTP lysed both human and murine PSMA⁺ target cells very effectively (Figure 3B and C). The same results were achieved with Pz-1-transduced PBL from two other patients with advanced, hormone-refractory prostate cancer (data not shown). Cytotoxicity was PSMA-specific, as Pz-1-transduced T cells did not kill PSMA-negative PC-3 or EL-4 cells any more than NTP-transduced lymphocytes. Back-

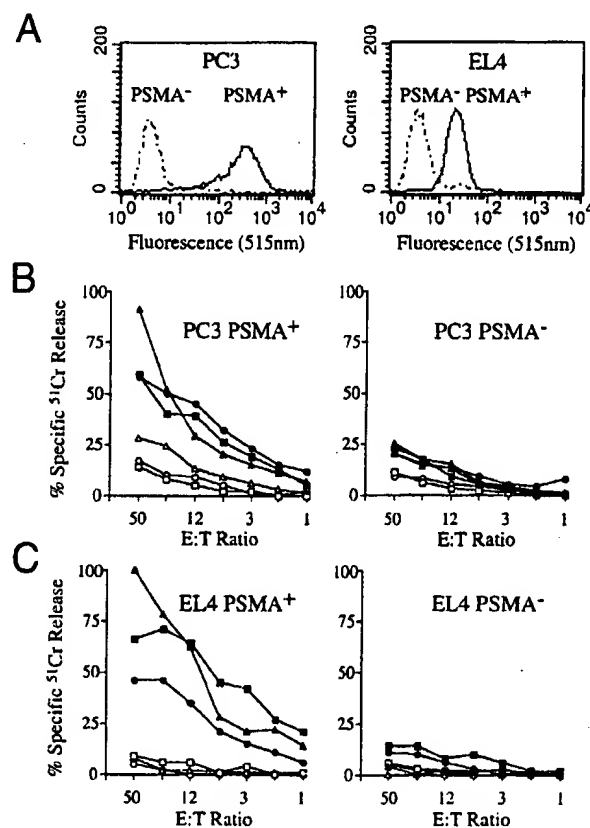


Figure 3. Pz-1-transduced prostate cancer patient T lymphocytes specifically lyse target cells expressing PSMA. (A) Left panel: FACS (Becton Dickinson & Co) analysis of wild type PC-3 cells (PSMA⁻) and PSMA-transduced PC-3 (PSMA⁺); PSMA⁻ cells (dotted line) coincide with PC-3 cells stained with the secondary GAM-FITC antibody alone (data not shown). Cells were stained with mAb J591 [8], followed by GAM-FITC antibody. Y axis: cell count; X axis: fluorescence (515 nm). Right panel: FACS (Becton Dickinson & Co) analysis of wild type EL-4 murine thymoma cells (PSMA⁻) and PSMA-transduced EL-4 cells (PSMA⁺). (B) Pz-1-transduced PBL specifically lyse PSMA⁺ target cells. PBL from three patients (Tables 1 and 2) were transduced with either Pz-1 or NTP [9]. Percentage of specific lysis is represented on the Y axis, effector to target ratio on the X axis. Targets are PC3-PSMA⁺ and PC3-PSMA⁻ (wild type), as described in (A, left panel). (C) Pz-1-transduced PBL specifically lyse PSMA⁺ EL-4 cells. Pz-1-transduced PBL of all patients effectively lysed PSMA⁺ targets. Background activity was variable on human target cells and uniformly low on murine target cells.

ground cytotoxicity levels varied on the human target cell lines but remained very low on the murine cells, suggesting that the allogeneic disparity between patient T cells and human target cell lines accounted for the higher background levels. The level of cytolytic activity did not closely correlate with the transduction rate achieved or the percentage of CD8⁺ in each case. Thus, elevated PSMA-specific cytotoxicity was obtained in Pz-1-transduced T cells in five of five prostate cancer patients, independent of their age and clinical stage of disease.

Pz-1 Signaling Causes Cytokine Secretion by Transduced PBL

To further assess the response of Pz-1-transduced primary T cells to PSMA, we investigated whether Pz-1⁺ PBL could release cytokines upon engagement with cell-bound PSMA. We established a cocultivation system in which transduced PBL are cultured on a monolayer of NIH3T3 fibroblasts engineered to express PSMA. NIH3T3 cells were transduced with retrovirus encoding PSMA, human B7.1 (CD80), or both PSMA and B7.1. Figure 4A shows the FACS (Becton Dickinson & Co) analysis of fibroblasts coexpressing PSMA and B7.1 (Latouche et al., unpublished observations). NIH3T3 cells expressing either PSMA or B7.1 alone showed the same respective fluorescence pattern (data not shown). Control NIH3T3 cells stained with either mAbs J591 (anti-PSMA) or BB1 (anti-B7.1) were negative, coinciding with the GAM^F peak (data not shown). T cells were cocultured in the absence of exogenous IL-2. Culture supernatants were sampled after 24, 48, and 72 hours and assayed for IL-2 and IFN- γ secretion. Pz-1-transduced T cells released significant amounts of IL-2 (Figure 4B) and IFN- γ (data not shown) 24 hours after exposure to PSMA⁺ feeder cells. IL-2 was barely detectable in the supernatant of Pz-1⁺ T cells cocultured with NIH3T3 cells expressing B7.1 alone or in the absence of

any feeder layer. However, IL-2 and IFN- γ (data not shown) secretion were strongly potentiated in the presence of both PSMA and B7.1, resulting in 10-fold higher IL-2 release after 24 hours (Figure 4B). Thus, cytokine secretion confirmed that B7.1-mediated signaling provided functional costimulation in Pz-1-transduced T cells stimulated by cell-bound antigen, suggesting that Pz-1 signaling synergized with B7.1-mediated costimulation. During the cocultivation, Pz-1⁺ PBL destroyed PSMA⁺ monolayers within 48 hours, whereas the PSMA⁺ B7.1⁺ monolayer remained intact during the entire 4-day cocultivation.

Discussion

Our results have several implications for the generation, expansion, and therapeutic use of T lymphocytes genetically targeted to a defined antigen. Using highly efficient retroviral-mediated gene transfer [9], we were able to investigate Pz-1 function in human primary CD4⁺ and CD8⁺ T cells.

First, we show that T cells of cancer patients can be genetically directed to recognize and lyse tumor cells that express the cell-surface antigen PSMA. The demonstration that an antigen-specific ζ chain fusion receptor is functional in cancer patient lymphocytes is an important step toward the implementation of adoptive cell therapies based on the genetic alteration of autologous polyclonal PBL. Indeed, defective TCR signaling has been observed in chronic tumor-bearing mice and cancer patients [10,11]. However, we found that the Pz-1 receptor was functional in T cells in five of five cancer patients, including three with advanced disease. These results suggest that T cell activation defects are either uncommon or bypassed by genetically modifying T cells with ζ chain fusion receptors. These results, therefore, alleviate our earlier concerns that findings in normal T cells might not extend to cancer patient cells.

All reports to date describing ζ chain fusions have focused on the antigen-specific killing imparted by the receptor (reviewed in Ref. [4]). However, the ability of ζ chain receptors to provide antigen specific helper functions has not been examined. We show that Pz-1 specifically elicited cytokine release when culturing the transduced lymphocytes on PSMA⁺ murine fibroblasts. This result indicates that the Pz-1 receptor provided a sufficient signal to trigger T-cell activation. However, the cytokine secretion was relatively weak and not sustained (Figure 4B). We therefore investigated whether providing costimulation to the transduced lymphocytes would increase their cytokine response. Indeed, whereas ζ chain signaling provides a powerful activation signal after engagement of the physiologic TCR, full T-cell activation typically requires a costimulatory signal [12]. Costimulation could act by amplifying the activation signal or offsetting putative proapoptotic signaling by the Pz-1 receptor.

On a practical level, our results also suggest a strategy to expand cells expressing an artificial TCR. *In vitro*, fibroblast monolayers that express antigen and B7 are very effective

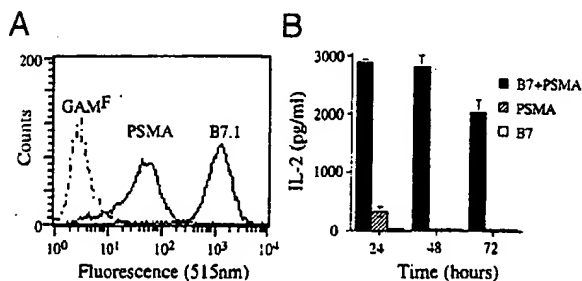


Figure 4. NIH3T3 fibroblast feeder cells coexpressing PSMA and B7.1 (CD80) direct cytokine release by Pz-1-transduced T cells. (A) Transduced NIH3T3 feeder cells were stained with mAb J591 as described in Figure 2 or with FITC-labeled mAb BB1 specific for B7.1. Y axis: cell count; X axis: PSMA or B7.1 signal. GAM^F: GAM-FITC antibody alone. PSMA and B7.1 indicate the fluorescence obtained for each stain. (B) PSMA induced IL-2 secretion by Pz-1⁺ T cells. Pz-1-transduced T cells were cocultured with NIH3T3 fibroblast feeder cells. Supernatants collected 24, 48, and 72 hours after the start of the coculture were assayed for IL-2 by ELISA. B7+PSMA, PSMA, B7, refer to the molecules expressed by the different fibroblast monolayers. Results are from quadruplicate experiments. Error bars represent 2 standard deviations.



as shown here. Different approaches can be envisaged to provide costimulation *in vivo*. One is to express B7.1 in tumor cells [13], providing that B7.1 can be expressed in a sufficient fraction of the tumor cells. Another is to provide antigen-dependent costimulation [5] by expressing in the T cells an antigen-specific CD28 fusion receptor. In this manner, contact with PSMA⁺ cells would provide CD28 signaling in the absence of the B7.1 or B7.2 ligands on tumor cells. Dendritic cells, which constitutively express B7.1, do not express native PSMA and thus could not appropriately stimulate the transduced T cells. To do so, the dendritic cells would have to be genetically engineered to express the intact cell-surface molecule [14].

Retroviral-mediated gene transfer is efficient in both CD4⁺ and CD8⁺ subsets of human T lymphocytes (Ref. [9]; Figure 1B). As the Pz-1 receptor signals for both the activation of cytotoxicity (Figures 2 and 3) and cytokine secretion (Figure 4B), it becomes envisagable to combine the adoptive transfer of PSMA-targeted CD4⁺ and CD8⁺ T cells. Cytokine release by the former should sustain the activity of the latter, in addition to exerting either proinflammatory and/or antitumoral functions. The expression of an artificial T-cell receptor that is not restricted by either MHC class I or class II creates the intriguing situation in which both CD4⁺ and CD8⁺ T lymphocytes could be activated side by side at the tumor site.

PSMA is expressed by normal prostate epithelial cells, and its expression is increased in prostate cancer, including metastatic and hormone-deprived cells. It is also expressed on the neovasculature of multiple carcinomas, the brush border of duodenal epithelium and salivary glands ([6–8] Chang et al., manuscript submitted). Thus, Pz-1 may be useful to target T cells to local and metastatic prostate cancer and tumor neovasculature of other solid tumors. Expression on normal tissues does not necessarily represent an obstacle to the therapeutic use of monoclonal antibodies [15,16]. Clinical studies are underway to define the biodistribution of the PSMA-specific J591 antibody *in vivo*.

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